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Detection of *Acinetobacter* spp. in Blood Cultures by an Improved Fluorescent *in Situ* Hybridization Assay

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Abstract

Fluorescent *in situ* hybridization (FISH) allows rapid detection of microorganisms. We aimed (i) to evaluate the sensitivity and specificity of FISH for the detection of *Acinetobacter* spp. in blood culture specimens and (ii) to test the simultaneous application of two genus-specific probes labeled with the same fluorochrome to increase the fluorescent signal intensity and improve the detection of *Acinetobacter* spp. Three hundred and twenty blood culture specimens were tested *via* both the conventional laboratory methods and FISH to detect *Acinetobacter* spp. The specimens were examined separately with each genus-specific probe Aci and ACA, and also using a mixture of the both probes Aci and ACA. In all examinations, probe EUB338 was used accompanied by Aci and ACA. The specificity of FISH was 100% (97.5% confidence interval [CI] = 98.7% – 100%). The sensitivity of FISH by the use of probe Aci was 96.4% (95% CI = 81.7% – 99.9%), whereas, the sensitivity of this technique by the use of probe ACA as well as by the combination of both probes Aci and ACA was 100% (97.5% CI = 87.7% – 100%). Moreover, simultaneous hybridization by probes Aci and ACA increased the fluorescent signal of *Acinetobacter* spp. cells to 3+ in 13 specimens. In conclusion, FISH, particularly using a combination of Aci and ACA, is a highly accurate method for the detection of *Acinetobacter* spp. in blood cultures. Furthermore, simultaneous hybridization by the both probes Aci and ACA can increase the fluorescent signal intensity of *Acinetobacter* spp. cells in some blood culture specimens and facilitate the detection of these microorganisms.

Key words: *Acinetobacter*, bacteremia, blood culture, FISH, simultaneous hybridization

Introduction

Acinetobacter spp. are aerobic, oxidase negative, and nonfermentative Gram-negative bacteria that have been reported to cause various nosocomial infections such as bacteremia (Phillips, 2015; Endo *et al.*, 2014). *Acinetobacter* bloodstream infection is typically associated with intravascular devices (Phillips, 2015). The mortality rate of *Acinetobacter baumannii* bacteremia can be 40.2% (Gu *et al.*, 2016). Development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *A. baumannii* is an increasing concern in the healthcare sector. In an investigation on *A. baumannii* isolates from a referral hospital in Southern Iran, 53% and 44% of isolates were identified as having MDR and XDR phenotypes, respectively (Alaei *et al.*, 2016). Use of appropriate antimicrobial drugs is thus crucial in the management of *Acinetobacter* spp. infections, particularly bacteremia. Detection of *Acinetobacter* spp. in blood culture specimens using conventional cultural and biochemical methods is time-consuming and requires at least two days, while, rapid detection of causative organism is essential for immediate selection of appropriate antibiotics and quick start of proper therapy of the patients. A rapid detection can improve prognosis and decrease the length of hospitalization (Peters *et al.*, 2006). Therefore, rapid detection of *Acinetobacter* spp. in blood cultures is required.

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Fluorescent *in situ* hybridization (FISH) using rRNA-targeted fluorescently labeled probes is a helpful rapid method that has been used for the identification of various microbes (Peters *et al.*, 2006; Tajbakhsh *et al.*, 2011; Poppert *et al.*, 2010; Tajbakhsh *et al.*, 2013b). Also, application of FISH using DNA probe for the rapid identification of *Acinetobacter* spp. from colony and blood culture specimens has been reported by Frickmann *et al.* (2011); in their study both the sensitivity and specificity of FISH were 100%. Although numerous reference strains and clinical isolates of *Acinetobacter* spp. and non-target organisms were tested *via* FISH by these authors, only seven *Acinetobacter*-positive blood culture specimens were found and investigated in their work (Frickmann *et al.*, 2011). However, for a more precise evaluation on the sensitivity of FISH for the detection of *Acinetobacter* spp. in blood cultures, further investigation using a higher number of *Acinetobacter*-positive blood culture specimens is required.

A probable limitation of FISH technique is the low signal intensity of some microbial cells that may make difficulties for the detection of microorganisms. A reason for the weak fluorescent signal is the low ribosome content found in some bacterial cells (Moter and Göbel, 2000; Zwirglmaier, 2005). Moreover, materials surrounding the bacteria in samples as well as blood cells such as erythrocytes and eosinophile granulocytes can exhibit a background fluorescence which may mask the specific fluorescent signal of microorganisms (Peters *et al.*, 2006; Moter and Göbel, 2000). One solution to enhance the specific fluorescent signal can be to use two or more specific probes labeled with the same fluorochrome and targeting different regions of the rRNA to increase the number of fluorescent molecules per microbial cell (Moter and Göbel, 2000; Zwirglmaier, 2005).

Our objectives in this study were (i) to evaluate the FISH for the detection of *Acinetobacter* spp. in blood culture specimens and (ii) to investigate the simultaneous application of two genus-specific probes labeled with the same fluorochrome in order to increase the fluorescent signal intensity and improve the detection of *Acinetobacter* spp. in these specimens.

Experimental

Materials and Methods

Bacterial strains and cell fixation. The American Type Culture Collection (ATCC) and the Persian Type Culture Collection (PTCC) reference strains, as well as other bacterial strains used in our investigation were *A. baumannii* (ATCC 19606 and three clinical isolates), *Acinetobacter calcoaceticus* (PTCC 1318), *Acinetobacter haemolyticus* (two clinical isolates), *Acinetobacter* spp. (five clinical isolates), *Stenotrophomonas maltophilia* (ATCC 13637 and six clinical isolates), *Pseudomonas aeruginosa* (PTCC 1707), *Pseudomonas* sp. (environmental isolate), *Microbacterium* (*Flavobacterium*) *aborescens* (ATCC 4358), *Flavobacterium* spp. (three clinical isolates), *Neisseria meningitidis* (ATCC 13090), *N. meningitidis* (PTCC 1507), *Brucella abortus* (S19 and one clinical isolate), *Brucella melitensis* (ATCC 23456), *Shewanella* sp. (environmental isolate), *Aeromonas* sp. (clinical isolate), *Plesiomonas shigelloides* (clinical isolate), *Vibrio parahaemolyticus* (ATCC 17802), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) (ATCC 14028), *Salmonella enterica* subsp. *enterica* serovar Typhi (*Salmonella* Typhi) (PTCC 1609), *Escherichia coli* (ATCC 8739), *Yersinia enterocolitica* (PTCC 1477), *Serratia marcescens* (clinical isolate), *Enterobacter aerogenes* (clinical isolate), *Citrobacter diversus* (clinical isolate), *Providencia rettgeri* (clinical isolate), *Proteus penneri* (environmental isolate), and *Streptococcus pneumoniae* (ATCC 49619). These strains were used to check the specificity of probes.

The bacterial strains outlined above were grown, harvested while in the exponential growth phase, and fixed with 4% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany) at 4°C for 1 h. The fixation protocol has been described elsewhere (Tajbakhsh *et al.*, 2008). All fixed bacterial strains were then examined *via* FISH, as explained below.

Blood culture specimens. This project was approved by the Ethical Committee of Bushehr University of Medical Sciences with reference number B-93-16-13. Between December 2014 and October 2015, a total of 320 positive blood culture specimens determined to contain Gram-negative bacteria or Gram-positive cocci by Gram staining, were collected from a major university hospital in the city of Bushehr, south west of Iran. The specimens were examined *via* conventional laboratory methods and FISH to detect genus *Acinetobacter*. Due to a tendency to retain crystal violet, *Acinetobacter* organisms may initially appear as Gram-positive cocci in direct smears made from blood culture specimens (Doughari *et al.*, 2011), and that is why the specimens containing Gram-positive cocci were also included in this study.

Conventional laboratory methods. An aliquot of each positive blood culture specimen was subcultured on blood agar (Merck, Darmstadt, Germany) and MacConkey agar (Merck, Darmstadt, Germany) plates and incubated for 24 h. Identification of the grown colonies was carried out by conventional laboratory methods such as Gram staining, culturing in triple sugar iron (TSI) agar (Merck, Darmstadt, Germany), oxidase, catalase, lysine decarboxylase, nitrate reduction, esculin

Table I
Probes used for FISH.

Probe	Sequence (5'-3')	Fluorochrome	Target	Reference
Aci	TTA GGC CAG ATG GCT GCC	Cy3	*Acinetobacter* spp.	(Frickmann *et al*., 2011)
ACA	ATC CTC TCC CAT ACT CTA	Cy3	*Acinetobacter* spp.	(Wagner *et al*., 1994)
EUB338	GCT GCC TCC CGT AGG AGT	Fluo	Bacteria	(Amann *et al*., 1990)

hydrolysis, indole, and motility tests (Doughari *et al.*, 2011), as well as DS-DIF-NONFERM (Yablonevaya, Nizhny Novgorod, Russia) or API 20 E (bioMérieux SA, Marcy-I'Etoile, France) kits. The DS-DIF-NONFERM was used for the identification of nonfermenters including *Acinetobacter* spp. The API 20 E was used for the identification of fermentative bacteria.

FISH. To fix the blood culture specimens, 200 µl of each specimen was mixed with 3 volumes of 4% paraformaldehyde and the next steps of the fixation procedure were performed as described previously (Tajbakhsh *et al.*, 2008).

The oligonucleotide probes used in the present study (Table I) were synthesized and 5'-labeled with fluorochromes Cy3 or Fluo (Metabion, Planegg/Steinkirchen, Germany). The probes Aci (Aci-16S 729) (Frickmann *et al.*, 2011) and ACA (Wagner *et al.*, 1994) that each targets a different position of the 16S rRNA of *Acinetobacter* spp., were used for the detection of the genus *Acinetobacter*. The 5' ends of the probes Aci and ACA were labeled with fluorochrome Cy3, which exhibits a red fluorescent signal. The probe EUB338, that targets and hybridizes a region of the 16S rRNA of almost all bacteria (Amann *et al.*, 1990), was 5'-labeled with fluorochrome Fluo, which emits a green signal. All of the control bacterial strains and blood culture samples were examined by FISH using three different mixtures of the probes on separate glass slides: (i) Aci-Cy3 and EUB338-Fluo, (ii) ACA-Cy3 and EUB338-Fluo, and (iii) Aci-Cy3, ACA-Cy3, and EUB338-Fluo.

The FISH procedure was performed as follows: 10 µl of each fixed control bacterial strain or each fixed blood culture sample were put on glass slides and air dried. For the dehydration, the slides were submerged for 3 min in each 50%, 80%, and absolute ethanol (Tajbakhsh *et al.*, 2008). In the hybridization step, specimens or bacterial strains were covered with 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.01% SDS, 30% formamide) containing a mixture of the probes. As mentioned above, each strain or blood culture specimen was tested separately with the three different mixtures of the probes: Aci and EUB338, ACA and EUB338, as well as Aci, ACA, and EUB338. The slides were then incubated at 46°C for 90 min in the moisture chambers for the hybridization. Subsequently, the slides were immersed into a washing buffer (20 mM Tris-HCl [pH 8], 0.01% SDS, 112 mM NaCl) and incubated at 48°C for 15 min. DNA was then stained with 1 µg/ml 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI; Roche, Mannheim, Germany) for 5 min. Afterwards, the slides were rinsed with phosphate buffered saline, left to air-dry, and mounted with a fluorescent mounting medium (DAKO, Glostrup, Denmark) (Tajbakhsh *et al.*, 2011; Moosavian *et al.*, 2007). The slides were observed and analyzed with an epifluorescence microscope (Nikon 80i, Tokyo, Japan) equipped with a DS-5Mc-L1 digital camera system. Microscopy was performed in a blinded manner by two investigators. The tests were carried out twice. In this study, the positive results of FISH were categorized based on the fluorescent signal intensity as follows: 1+ (weak fluorescent signal), 2+ (moderate fluorescent signal), and 3+ (strong fluorescent signal).

Analysis of assay. The results of FISH were compared with the results of the conventional laboratory methods of identification. The sensitivity and specificity of FISH were calculated with the formulas $(a/(a+c)) \times 100$ and $(d/(b+d)) \times 100$, respectively, where a = true positive, b = false positive, c = false negative, and d = true negative. Ninety five percent confidence interval (95% CI) was calculated using Exact Binomial method. If calculated sensitivity or specificity was 100%, one-sided 97.5% confidence interval was calculated using the same method. Statistical analyses were performed using StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP.

Results

A set of bacterial strains was used to check the specificity of the probes (Table II). The probe EUB338 hybridized all bacterial strains. However, both the probe Aci and ACA hybridized exclusively to *Acinetobacter* species but not to any of the negative controls, which indicates the high specificity of these probes (specificity 100% [97.5% CI = 89.1% – 100%]).

In this project, 320 positive blood culture specimens were tested by both conventional laboratory identification and FISH. By conventional culturing, *Acinetobacter* spp. were detected in 28 of 320 specimens. FISH using probe Aci detected *Acinetobacter* spp. in 27 of these 28 *Acinetobacter*-positive blood culture specimens, whereas FISH by the use of probe ACA, and also by

Table II
Examination of bacterial strains *via* FISH.

Bacteria	Source	Results of hybridization with probe		
		EUB338	Aci	ACA
A. baumannii	ATCC 19606	+	+	+
A. baumannii	3 clinical isolates	+	+	+
A. calcoaceticus	PTCC 1318	+	+	+
A. haemolyticus	2 clinical isolates	+	+	+
Acinetobacter spp.	5 clinical isolates	+	+	+
S. maltophilia	ATCC 13637	+	–	–
S. maltophilia	6 clinical isolates	+	–	–
P. aeruginosa	PTCC 1707	+	–	–
Pseudomonas sp.	Seawater	+	–	–
M. aborescens	ATCC 4358	+	–	–
Flavobacterium spp.	3 clinical isolates	+	–	–
N. meningitidis	ATCC 13090	+	–	–
N. meningitidis	PTCC 1507	+	–	–
B. abortus	S19	+	–	–
B. abortus	clinical isolate	+	–	–
B. melitensis	ATCC 23456	+	–	–
Shewanella sp.	Seawater	+	–	–
Aeromonas sp.	clinical isolate	+	–	–
P. shigelloides	clinical isolate	+	–	–
V. parahaemolyticus	ATCC 17802	+	–	–
S. Typhimurium	ATCC 14028	+	–	–
S. Typhi	PTCC 1609	+	–	–
E. coli	ATCC 8739	+	–	–
Y. enterocolitica	PTCC 1477	+	–	–
S. marcescens	clinical isolate	+	–	–
E. aerogenes	clinical isolate	+	–	–
C. diversus	clinical isolate	+	–	–
P. rettgeri	clinical isolate	+	–	–
P. penneri	Seawater	+	–	–
S. pneumoniae	ATCC 49619	+	–	–

the mixture of both probes ACA and Aci, could detect *Acinetobacter* spp. in all of the mentioned 28 specimens. In other words, probe Aci, but not ACA, failed to detect *Acinetobacter* sp. in one specimen. The remaining 292 samples were negative for *Acinetobacter* spp. according to both the conventional identification and FISH. Therefore, based on the results of our study, the sensitivity of FISH for the detection of *Acinetobacter* spp. in blood culture specimens using the probe Aci was 96.4% (95% CI = 81.7% – 99.9%), whereas, the sensitivity of this technique by the use of probe ACA and by the mixture of both probes ACA and Aci was 100% (97.5% CI = 87.7% – 100%). The specificity of FISH for the detection of *Acinetobacter* spp. in blood cultures was 100% (97.5% CI = 98.7% – 100%).

Furthermore, in this study, we attempted to improve the specific fluorescent signal of *Acinetobacter* organisms. The simultaneous hybridization by two genus-specific probes, Aci and ACA, labeled with the same fluorochrome (Cy3), increased the fluorescent signal intensity of *Acinetobacter* spp. cells from 1+ or 2+ to 3+ in 13 of 28 *Acinetobacter*-containing blood culture specimens and facilitated the observation and detection of these microorganisms (Fig. 1). No change in fluorescent signal intensity was observed in the remaining 15 *Acinetobacter*-containing blood culture specimens by application of the mixture of the probes Aci and ACA.

It should be noted that in three of the 28 *Acinetobacter*-containing blood cultures, *Acinetobacter* spp. initially appeared as Gram-positive cocci in direct smears prepared from the specimens. However, the organisms in these three specimens were successfully identified as *Acinetobacter* spp. *via* both FISH and further conventional biochemical identification.

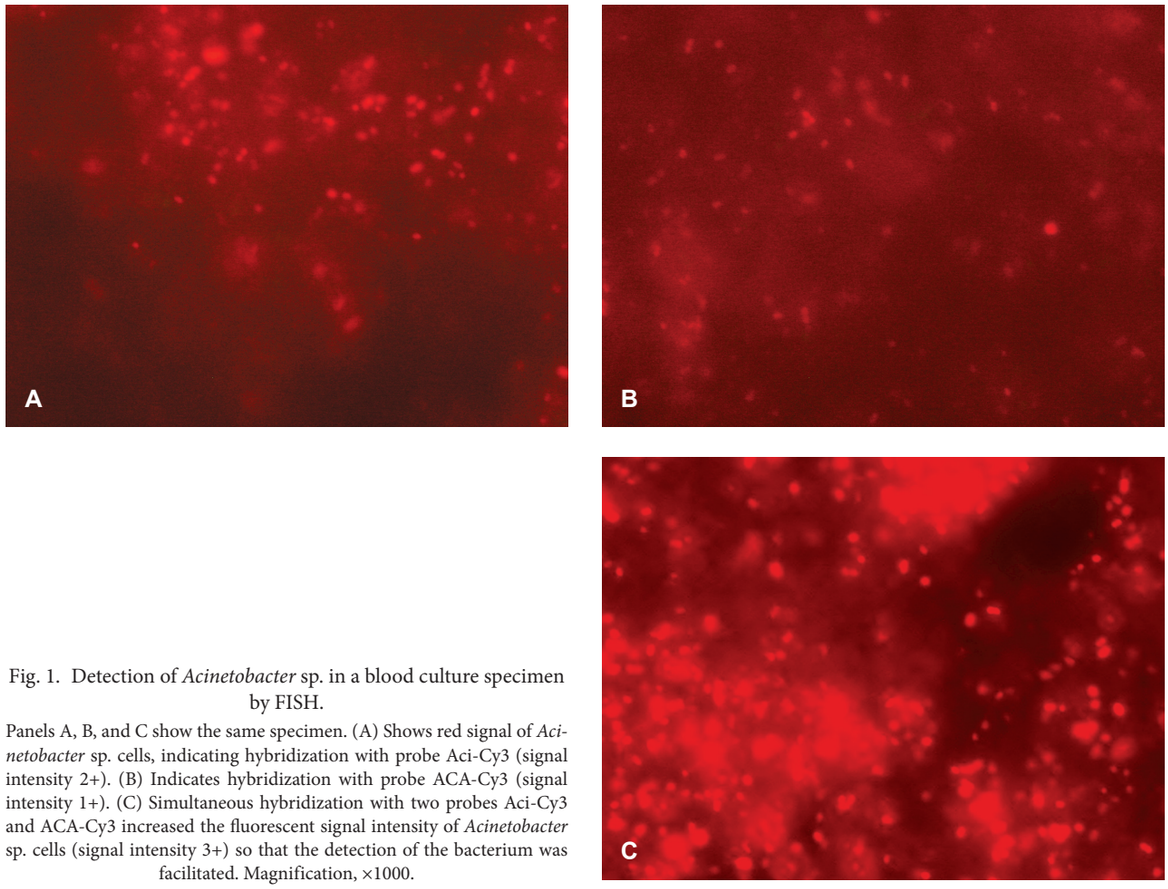

Fig. 1. Detection of *Acinetobacter* sp. in a blood culture specimen by FISH.

Panels A, B, and C show the same specimen. (A) Shows red signal of *Acinetobacter* sp. cells, indicating hybridization with probe Aci-Cy3 (signal intensity 2+). (B) Indicates hybridization with probe ACA-Cy3 (signal intensity 1+). (C) Simultaneous hybridization with two probes Aci-Cy3 and ACA-Cy3 increased the fluorescent signal intensity of *Acinetobacter* sp. cells (signal intensity 3+) so that the detection of the bacterium was facilitated. Magnification, ×1000.

Discussion

Reducing the time required for identification of microorganisms in positive blood cultures is important to enable satisfactory pathogen-based antibiotic therapy at an early phase and to improve outcome (Peters *et al.*, 2006; Frickmann *et al.*, 2011). In this paper, we have designed a study to evaluate the FISH technique and to use of a combination of two genus-specific probes to improve the FISH procedure for the detection of *Acinetobacter* spp. in blood culture samples.

In this study, the oligonucleotide probes Aci and ACA were used to identify genus *Acinetobacter*. In the study conducted by Frickmann *et al.* (2011), the probe Aci which was tested with many bacterial species, correctly identified all *Acinetobacter* spp. and excluded all non-target bacterial species, and therefore found to be highly specific. In the present study, we added more bacterial strains including *Flavobacterium* spp., *B. abortus*, *B. melitensis*, *N. meningitidis*, *Shewanella* sp., *Aeromonas* sp., *P. shigelloides*, *V. parahaemolyticus*, *P. rettgeri*, and *S. pneumoniae*, that may cause bacteremia and be present in blood cultures (Hall, 2015; Wellinghausen *et al.*, 2006; Carroll and Hobden, 2016; Hochedez *et al.*, 2010; Chen *et al.*, 2013; Cheng *et al.*, 2015; Choi *et al.*, 2015; Tajbakhsh *et al.*, 2013a) and were not examined by Frickmann *et al.* (2011). These strains were not hybridized with probe Aci and thus we confirmed the high specificity of this probe for the detection of *Acinetobacter* spp. The following reasons help to explain why these strains were added in our study to check probe specificity. *Flavobacterium* spp. are isolated from a few blood culture samples in the city of Bushehr. Also, brucellosis is prevalent in our geographic area and blood is one of the specimens in which *Brucella* spp. are often found. Consequently, the correct negative results with these bacteria were important for us. The reason for examination on *N. meningitidis* was that *Acinetobacter* spp. resemble *Neisseria* spp. on conventional smears, so that *Acinetobacter* spp. recovered from bacteremic patients have been mistaken for *N. meningitidis* (Carroll and Hobden, 2016); however, Aci could successfully differentiate *Acinetobacter* spp. from this bacterium. Regarding examination on *V. parahaemolyticus*, *Aeromonas* sp., and *Shewanella* sp., it should be mentioned that Bushehr is a seaport with a vast coastal region and its people have much contact with microorganisms in marine water. Since *Vibrio* spp., *Aeromonas* spp., and *Shewanella* spp. are commonly found in aquatic environment such as marine water (Hochedez *et al.*, 2010; Janda and Abbott,

2014), it was needed to use of these bacteria as negative control for the probe. Also, because seafood is a natural reservoir of *P. shigelloides* (Chen *et al.*, 2013), and occupational exposure can be a source of bacteremia for fish handlers, we also decided to test the probe on this organism. Furthermore, although *S. pneumoniae* is a Gram-positive organism, it was used for the evaluation of probe specificity. We previously showed that the oligonucleotide probes can penetrate into the *S. pneumoniae* cells without enzymatic treatment, *i.e.*, the FISH procedure for this bacterium is similar to the procedure for Gram-negative organisms (Tajbakhsh *et al.*, 2013a). The probe Aci also produced a correct negative result with *S. pneumoniae* as mentioned above. It should be emphasized that the results of the examination of bacterial strains with probe ACA were same to the results of the probe Aci, and both probes were highly specific for the detection of *Acinetobacter* spp. ACA was developed by Wagner *et al.* (1994) and applied for *in situ* monitoring of *Acinetobacter* spp. in activated sludge. We used the probe ACA in the field of clinical microbiology.

Three hundred and twenty blood cultures were examined to evaluate the sensitivity and specificity of FISH for the detection of *Acinetobacter* spp. No false-positive results were observed and the specificity of FISH was 100%. By conventional identification, 28 specimens were positive for *Acinetobacter* spp., of which 27 specimens were FISH positive using probe Aci. Thus, the sensitivity of FISH in blood culture specimens by the use of probe Aci was 96.4%. Our results are close to the results of the investigation performed by Frickmann *et al.* (2011); in their work the sensitivity and specificity of FISH using probe Aci were 100%. In the present study, no false-negative results were observed by the use of probe ACA, as well as by the combination of probes ACA and Aci, and thus a 100% sensitivity was achieved. FISH is therefore a highly accurate method for the detection of *Acinetobacter* spp. in positive blood cultures. A benefit of the simultaneous application of probes Aci and ACA is that if one probe failed to identify *Acinetobacter*, the organism may be identified by the other one.

Wong *et al.* (2007) used DNA probe for the detection of *Acinetobacter* spp. from positive blood cultures. Although they did not state the number of blood culture specimens used for the evaluation of FISH, both sensitivity and specificity have been reported to be 100%. Our results are in accordance with the results of the investigation performed by Wong *et al.* (2007). Also, the potential of peptide nucleic acid (PNA) probe for the detection of *Acinetobacter* spp. from blood cultures has been shown (Peleg *et al.*, 2009). However, PNA probes are expensive.

There are reports concerning the other rapid methods for the detection of *Acinetobacter* spp. in positive blood cultures. Rapid identification of *A. baumannii*, *A. nosocomialis*, and *A. pittii* with a multiplex PCR assay showed a sensitivity of 92.4% and specificity of 98.2%. False-positive results were observed in this method so that blood culture samples containing bacteria such as *Aeromonas hydrophila*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, or *Pseudomonas putida* were detected as *Acinetobacter*-positive by multiplex PCR. Moreover, false-negative results of multiplex PCR were reported, however, altogether it has been reported as a convenient assay (Chen *et al.*, 2014). Also, rapid and accurate identification of *A. baumannii* in positive blood cultures using matrix-assisted laser desorption-ionization time- of-flight mass spectrometry (MALDI-TOF-MS) has been shown (Bazzi *et al.*, 2017), but it is expensive and requires specific equipment.

In this investigation, the FISH assay was improved by the combined use of two probes Aci and ACA, so that the intensity of fluorescent signal of *Acinetobacter* spp. in 13 blood cultures was increased and detection of the organism was facilitated. For this procedure, careful selection of probe sequences should be considered in order that the probes target to independent sites in the rRNA molecule and also probe-probe interaction should not occur because cross-hybridization of probes to each other results in reduced signals (Lee *et al.*, 1993). The probes Aci and ACA have the mentioned characteristics, *i.e.*, they (i) target independent sites and (ii) do not bind to each other. Meanwhile, it should also be said that combination of the probes Aci and ACA did not show any adverse effect on the FISH results. All of these conditions support the idea of using the combination of Aci and ACA for the improving the fluorescent signal of *Acinetobacter* spp. cells. The organism in the remaining 15 *Acinetobacter* – containing specimens emitted a strong fluorescent signal by hybridization with each probe, and no change in signal intensity was observed in these 15 specimens by combination of Aci and ACA. The lack of a background fluorescence in these 15 specimens might be the reason for the exhibition of a strong specific fluorescent signal of *Acinetobacter* spp. cells, even with each probe alone. In other studies, application of probe combinations to increase the signal intensity of natural planktonic bacteria (Lee *et al.*, 1993), *Desulfobacter hydrogenophilus* (Amann *et al.*, 1990), and *P. aeruginosa* (Hogardt *et al.*, 2000) has been reported.

In our study, *Acinetobacter* organisms appeared as Gram-positive cocci in direct smear prepared from three blood cultures. This is an important point and may influence on antimicrobial management and lead to administration of inappropriate antibiotics, because blood culture Gram stain results are used to guide initiation of antimicrobial regimens (Munson *et al.*, 2003).

However, FISH correctly detected *Acinetobacter* spp. in these three specimens on the same day. Therefore, we strongly recommend the application of *Acinetobacter* probes for Gram-positive cocci observed in blood cultures besides for Gram-negative bacteria. Such blood cultures containing Gram-positive cocci were not examined in the previous studies (Frickmann *et al.*, 2011; Wong *et al.*, 2007).

In conclusion, FISH, particularly by the use of a combination of probes Aci and ACA, is a highly accurate technique for the detection of *Acinetobacter* spp. in positive blood cultures. A benefit of the simultaneous application of the probes Aci and ACA is that if one probe failed to identify *Acinetobacter*, there is still a possibility for the other one to identify the organism. Furthermore, simultaneous hybridization by the both probes Aci and ACA can increase the fluorescent signal intensity of *Acinetobacter* spp. cells at least in some blood culture specimens and facilitate the observation and detection of these microorganisms. FISH can also be a method of rapid identification when *Acinetobacter* organisms appear as Gram-positive cocci in direct smears from blood cultures.

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